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Effects of field-relevant concentrations of clothianidin on larval development of the butterfly *Polyommatus icarus* (Lepidoptera, Lycaenidae)

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Abstract

Arable field margins are often sown with wild flowers to encourage pollinators and other beneficial or desirable insects such as bees and butterflies. Concern has been raised that these margins may be contaminated with systemic pesticides such as neonicotinoids used on the adjacent crop, and that this may negatively impact on beneficial insects. The use of neonicotinoids has been linked to

butterfly declines, and species such as the common blue butterfly (*Polyommatus icarus*) that feed upon legumes commonly sown in arable field margins, may be exposed to such toxins. Here, we demonstrate that the larval foodplants of *P. icarus* growing in an arable field margin adjacent to a wheat crop treated with the neonicotinoid clothianidin, not only contain the pesticide at concentrations comparable to and sometimes higher than those found in foliage of treated crops (range 0.2 to 48 ppb), but remain detectable at these levels for up to 21 months after sowing the crop. Overall, our study demonstrates that non-target herbivorous organisms in arable field margins are likely to be chronically exposed to neonicotinoids. Under laboratory conditions, exposure to clothianidin at 15ppb (a field-realistic dose) or above reduced larval growth for the first 9 days of the experiment. Although there was evidence of clothianidin inducing mortality in larvae, with highest survival in control groups, the dose-response relationship was unclear. Our study suggests that larvae of this butterfly exhibit some deleterious sublethal and sometimes lethal impacts of exposure to clothianidin, but many larvae survive to adulthood even when exposed to high doses.

Introduction

British butterfly and moth species have been in steep decline since formal recording began in 1976, with 70% of species declining in occurrence, and 57% declining in abundance.[1] Species found on farmland appear to be faring particularly badly with nearly a 40% decline since 2005 in the **abundance** of English butterfly species alone, despite significant conservation **investment in** agri-environment schemes.[1–3] Similar patterns of decline have been observed around the globe, at least in locations where populations are accurately monitored.[4] The intensification of farming over the last few decades has led to a loss of habitat for wildlife,[5] and given that greater numbers of butterflies and butterfly species have been found on organic farms compared to conventional farms,[6,7] this suggests that chemical inputs are contributing to declines of Lepidoptera. **However, as organic farms may have a greater abundance and diversity of food plants in their field boundaries,[6] and greater species richness caused by an increase in landscape diversity within each organic field,[24] the contribution of chemicals may not be the sole difference between the two farm types/which would also contribute to the insect community.**

Neonicotinoid pesticides, alongside glyphosate, currently dominate the global pesticide market, and are used more than any other class of insecticides.[8] The current prophylactic use of neonicotinoids as seed dressings on many arable crops[9], combined with their persistence, solubility in water and systemic action in plants, presents a large-scale risk of neonicotinoid contamination of non-target plants. This includes the vegetation, pollen and nectar of semi-natural areas surrounding arable crops,[10–12] and hence poses a risk to non-target organisms living in these areas. In recent years, the majority of attention on neonicotinoid toxicity has been focussed on the risks to bees, as these are economically important pollinators of food crops. Semi-field and laboratory studies suggest that exposure of adult bees to field-relevant doses can impair pollen collection, increase worker mortality, reduce the production of new queens and weaken the bee's immune system.[13–16] However, this gives little insight into the impact of these compounds on other non-target groups.

Research into the effect of neonicotinoids on Lepidoptera has focussed on their effectiveness against moth larvae typically regarded as pests (reviewed in Pisa *et al*. 2015). These studies have found a wide range of interspecific tolerances to pesticides, with some moth species up to 100 times more sensitive than others.[18] Of the few studies that have looked at the impact of neonicotinoids on non-target Lepidoptera, the focus has been on the effects of acute exposure on larvae,[19,20] though in the field we would expect moth and butterfly larvae to be exposed throughout the duration of their development.[12]

Two recent studies found neonicotinoid use to be significantly negatively correlated with long-term butterfly population declines, both in the UK[3] and in California,[21] though it is not clear whether this is due to larval exposure, adult exposure, or both. Relatively few data exist providing field-relevant contamination levels in non-target vegetation**,** but those studies that do,[11,22–25] show that neonicotinoid concentrations overlap with lethal concentrations for some insect species.[12]

A recent review has suggested that there is a clear need for studies on the impact of neonicotinoids on non-target butterflies,[17] in particular those species, such as *Polyommattus icarus* (Lycaenidae, (Rottemburg, 1775), which inhabit agricultural landscapes and are at risk of exposure to neonicotinoids.[12] Here, we seek to address this gap in knowledge by first establishing typical vegetative contamination levels of *P. icarus'* food plants in the field, and secondly experimentally testing the effect of field-realistic doses of clothianidin via oral exposure on the mortality and development of *P. icarus* larvae.

Materials and Methods

Field Study

Environmental sampling

~~Twenty samples of *Lotus corniculatus* and *Trifolium hybridum* (Fabaceae), both common larval foodplants of *P. icarus*, were collected from Hope Farm, Cambridgeshire, UK, **from two metre wide**~~

~~**pollen and nectar** margins specifically managed for wildlife. The fields had been planted with wheat treated with Redigo Deter© (active ingredients: 50 g/L prothioconazole and 250 g/L clothianidin[11]). Margins were sampled either 10 months (Field A) or 21 months (Field B) from the time clothianidin treated winter wheat had been sown. The plants of field A were sown adjacent to contaminated soil, and the plants of field B were sown directly into contaminated soil **that had previously been sown with winter wheat**. Prior to this, these fields had never been exposed to neonicotinoids either through direct soil treatment or the growing of neonicotinoid treated crops (Figure S1).~~

The field study was based at Hope Farm, Cambridgeshire, UK, and was carried out over a 2-year period from September 2013 to August 2015. Prior to this study these fields had never been exposed to neonicotinoids either through direct soil treatment or the growing of neonicotinoid treated crops. In September 2013 Field A was planted with untreated winter wheat and Field B was planted with winter wheat treated with Redigo Deter© (active ingredients: 50 g/L prothioconazole and 250 g/L clothianidin[11]). No pollen and wildlife margins were created at this point as both fields were planted to their edges with wheat. In September 2014 a field margin of two meters was created around both fields and planted with sainfoin *Onobrychis viciifolia*, winter vetch *Vicia villosa*, birdsfoot trefoil *Lotus corniculatus*, Lucerne *Medicago sativa*, alsike clover *Trifolium hybridum* and dwarf amenity ryegrass *Lolium perenne*, to create a pollen and nectar margin. In October 2014, Field A was sown with untreated wheat and Field B with neonicotinoid treated wheat. Therefore, the margin of Field A would be growing alongside a treated crop and Field B's margin was planted in contaminated soil from the treated wheat planted in the first year. Figure 1 summarises the planting schedule.

On 15th August 2015, twenty samples of *Lotus corniculatus* and *Trifolium hybridum* (Fabaceae), both common larval foodplants of *P. icarus*, were collected from **the pollen and nectar** margins of each field. Two composite samples per plant species were taken at each of the 10 equidistant sampling

111 points along the margin of the two fields. **Thus, the margins were sampled after 10 and 21 months**
112 **after neonicotinoid exposure for 10 Field A and Field B respectively.**

113 Two grams of leaves were taken from all parts of multiple plants and were stored in plastic bags
114 lined with aluminium foil at -80^{o}C until time for sample preparation. One gram of sample foliage was
115 pulverised with a mortar and pestle in liquid nitrogen and analysed for the presence of
116 neonicotinoids (clothianidin, thiamethoxam, imidacloprid, acetamiprid and thiacloprid) with ultra-
117 high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) (following
118 methods described in detail in Botías *et al*. 2016). Samples were subsequently re-extracted from *L.*
119 *corniculatus* **and *T. hybridum*** at the sites which had shown high levels of clothianidin in clover to
120 confirm the high levels of clothianidin found in the *T. hybridum* samples and the low concentrations
121 found in the *L. corniculatus* samples. Where these levels differed in the second extract, the lower
122 results were used to calculate means.

123 ***Laboratory-based study***

124 *Experimental contamination of vegetation*

125 Individual 6cm pots of *T. repens* (0.2g of seed per pot) (Crocus, Surrey, England) were sown in
126 multipurpose growing medium (50g per pot) (B&Q, Brighton, United Kingdom, own brand) and
127 grown under greenhouse conditions. *Trifolium repens* was grown instead of the *T. hybridum* that had
128 been sampled in the field due to space constraints (it is a much smaller plant). Pots were placed onto
129 a deep plastic tray, through holes cut in a cardboard spacer which protected the watering solution
130 from light exposure, to minimise UV-degradation of the pesticide.

131 Clothianidin (analytical grade; Sigma-Aldrich, Gillingham, UK) solutions of 0ppb (control), 5ppb,
132 15pp, 50ppb and 500ppb were made up on alternate days from a stock solution diluted with acetone
133 stored in a -80 ^{o}C freezer. Solutions were watered directly into the tray so that no solution touched
134 the leaves.

Each treatment group **tray** was watered with 500ml each time. Plants were kept in the contamination tray for 2 weeks before the foliage was presented to the larvae. Seeds were continuously sown and new pots of *T. repens* were added to the contamination trays daily to ensure there was a continuous supply of contaminated foliage available that had been exposed for two weeks. Effective concentration levels in the leaves were analysed by taking a composite sample of foliar tissue from each treatment group two weeks after the initial dosing of the plants, and analysed using the same method that was used for the farm samples.

Obtaining eggs

Female *P. icarus* were captured at two different sites in Brighton, East Sussex (50.860343, -0.120088 and 50.869376, -0.085992) between the 7th and the 10th June 2016. These individuals were kept in 6L plastic aquariums in a temperature controlled laboratory (23^{o}C). Sponges soaked in a mixture of orange sports drink (Asda own brand, Asda, UK), simple syrup (a thick sugar liquid made from equal measures of water and caster sugar) and soy sauce (Kikkoman®); approximately 14: 1: 0.05 were provided for a food source. *L. corniculatus* seeds were sown in 12cm pots with a multipurpose growing medium (B&Q, Brighton, United Kingdom, own brand), and were provided as oviposition substrate

The plants were replaced every three days and eggs were counted. These plants were put into a holding aquarium with the same conditions and temperature. Once ~200 eggs had been laid within a 2-day period, these plants were placed in a climate control chamber at 24^{o}C, with 70% humidity to stimulate egg hatching. Eggs were obtained from 34 females in total.

Exposure of larvae

Seven day old larvae (from date of hatching) were randomly assigned across the 5 treatment groups into individual 9cm petri dishes lined with moistened filter paper; half of the lid was covered with black tape to provide shading. These were kept in a growth chamber at 70% humidity, 25^{o}C and a

16:8 hr day : night cycle [26]. There were 30 replicate larvae per group. Larvae were fed *ad libitum* with leaves of *T. repens* sampled from plants that had been in the contamination tray for exactly two weeks. The stems of the leaves were inserted into 1.5ml Eppendorf tubes with a pierced cap filled with mineral water (Asda own brand, Asda, UK) to prevent the leaves from wilting, and leaves were replaced every three days. After larval measurements had been taken (see below), using a soft paintbrush the larvae were carefully placed back into the petri dish, on fresh moistened filter paper.

Monitoring development of larvae

Measurements were taken every three days for a total period of 57 days. Larval length and width were measured to the nearest 0.01 mm using a microscope with graticule. Time to pupation was also recorded, and death was determined either when the larvae failed to respond when gently touched with the paintbrush or when there was cessation of food consumption for 5 days or more. The development time to specific instars could not be monitored accurately due to the resting habit of Lycaenidae larvae tucking their head capsule underneath their bodies, which meant that head capsule width could not be recorded without disturbing the individual.

As soon as the first larva had pupated, larvae were checked twice a day (09:00 and 18:00) to record pupation date. Pupae were left for 24 hours after pupation so that they could harden, before being weighed on a 0.001g resolution balance (Precisa 125A, Newport Pagnell, Bucks, UK) and then were placed in clear plastic cups lined with dampened paper towel inside the growth chamber. They were checked twice a day (09:00 and 18:00) for emergence. Once emerged, adults were left in situ for either 9 or 15 hours (until the next check time) in order to allow for their wings to fully unfurl. They were then killed by placing them back in to their plastic cups and into a -80^{o}C freezer until they were weighed on a 0.001g resolution balance.

Data Analysis

All analyses were performed in SPSS (IBM SPSS Inc., v. 22, USA).

Environmental samples

A Mann-Whitney U test was used to determine if there were differences in clothianidin and thiamethoxam concentrations between *T. hybridum* and *L. corniculatus*, **and between the experimental fields**. Due to dissimilar distribution shapes, rank means of contamination levels in foliage are reported. To perform the statistical analyses, all concentrations that were over the limits of detection (≥MDL) but below the limits of quantification (<MQL) were assigned the value considered as the MDL in each case (Table 1). Concentrations below the MDL were considered to be zero[12].

Laboratory study

Calculations investigating the impact of clothianidin on larval size were performed on a subset of data which excluded time points at which pupation had started to occur. Larval size (mm^2) across treatment groups was compared using repeated measures ANOVA with the treatment-by-time interaction as the primary effect of interest. Data were log-transformed in order to meet the assumptions of normality (as tested by the Shapiro-Wilk statistic and visual inspection of Q-Q plots) and also tested for homogeneity of variances prior to analysis. The assumption of sphericity (as defined by Mauchly's statistic) was not met for data from any treatment group, therefore Greenhouse-Geisser adjustments were made to correct the ANOVA and it is this adjusted p-value that is reported. Further pairwise comparisons were examined using Dunn's procedure with a Bonferroni correction for multiple comparisons.

For analysis of larval survival, all larvae that reached the pupal stage were defined as survivors, irrespective of whether they later successfully completed metamorphosis[27]. Survival of the larvae across the treatment groups was analysed using Kaplan-Meier survival analysis, and the log-rank test

with a Bonferroni correction was applied to test for differences between survival distributions. Once individuals reached pupation they were treated as 'censored data'. Censored data (i.e. the number of larvae reaching pupation) across treatment groups were dissimilar and are therefore reported (Table 2). Four larvae were still alive at the end of the experiment (11th August) (15ppb n=1, 50ppb n=3) and were excluded from the survival analysis.

Generalized Liner Models (GLM) were used to analyse pupal stage duration, adult weight, pupal weight and time taken to reach pupation with treatment and sex as the predicting factors. Normal error distribution was stipulated for pupal weight and adult weight analysis, and a Poisson distribution with a log link function was used for pupal stage duration and time taken to reach pupation due to departures from normality as defined by visual inspection of Q-Q plots. We first fitted full models and systematically omitted interaction terms if they did not increase model fit. Models were fitted to the data using Akaike Information Criterion (AIC).

Results

Environmental samples - Vegetation contamination and residue analysis

Clothianidin

Overall clothianidin residues in field margin plants ranged from 0 – 48 ng/g (Table 1). Levels found in *L. corniculatus* were higher in field B, where time between sowing and sampling was 21 months, than field A where time between sampling and sowing was less at 10 months (Mann Whitney U test: U= 86, z= 3.058, p= 0.005; mean rank: field A – 6.9, field B – 14.1, mean ± SD: field B – 0.30 ± 0.31 ng/g, field A – 0.02 ± 0.06 ng/g). Levels of clothianidin in field A were much higher in *T. hybridum* than those found in *L. corniculatus* (6.31 ng/g ± 14.17, 0.02 ng/g ± 0.06, respectively (Table 1); Mann Whitney U test: U= 86.5, z= 3.069, p= 0.004; mean rank: *T. hybridum* – 6.85, *L. corniculatus* – 14.15). There was no difference in clothianidin levels between species in field B (Mann Whitney U test, U=

67, z=1.353, p= 0.218). There was no statistical difference of clothianidin levels in *T. hybridum* between fields A and B (Mann Whitney U test: U=58.5, z= 0.667, p= 0.529). Frequency of clothianidin detection was also higher in field B (*L. corniculatus* - 80%, *T. hybridum* – 80%) compared to field A (*L. corniculatus* - 10%, *T. hybridum* – 80%).

Thiamethoxam

Foliar thiamethoxam residues ranged from 0 to 0.54 ng/g in field A which had the shortest time between sowing and sampling. Residue levels were significantly higher in field A than in field B (Mann Whitney U test: U= 5, z= -3.727, p= <0.001; mean rank: field A – 15, field B – 6). In field B, thiamethoxam was only detected in *L. corniculatus* (0.01 ± 0.03 ng/g, frequency of detection: 10%) with levels in *T. hybridum* being lower than the method detection limit (MDL; ≤0.1 ng/g)). Similar to clothianidin, frequency of detection of thiamethoxam was also higher in *T. hybridum* (Field A: (frequency of detection in *L.* corniculatus – 60% and *T. hybridum* – 90%).

If the results from both margins are combined to provide a more general field-realistic contamination overview irrespective of the time when the treated seed was planted, the mean clothianidin levels found in *T. hybridum* are 9.04 ng/g ± 15.22 (n=20), and 0.16 ng/g ± 0.26 in *L. corniculatus* (mean ± SD). Imidacloprid, acetamiprid and thiacloprid were not detected in the samples.

Larval survival

We found that our laboratory based *T. hybridum* leaf contamination method produced very similar concentrations in the foliage to those that were in the solutions (Mean result, n=2 of each treatment group: Control-0 ng/g; 5 ng/g -5 ng/g; 15 ng/g -14.5 ng/g; 50 ng/g -53.9 ng/g; 500 ng/g -439.1 ng/g).

Individuals in the 500ppb group experienced higher levels of mortality than the lower, field-realistic treatment groups (Kaplan-Meier analysis, **log rank: $\chi^2(4)=11.211$, p= .024 (Figure 2)),** with a clear

drop in survival at the start of the experiment (day 9). Post-hoc pairwise comparisons (Kaplan-Meier analysis, pairwise log-rank tests) highlighted significant differences between the control and 500ppb ($\chi^2(1)= 5.337$, p= 0.021) and 15ppb and 500ppb ($\chi^2(1)=$**5.337**, p= 0.01**8**. Percentage survival was lowest in the 50ppb treatment group (63%), with the greatest survival in the control group (90%) (Table 2).

Larval development

Three larvae in the 50ppb group and one individual in the 15ppb group were still alive at the end of the experiment, **the rest of the larvae had either died or pupated.** These larvae still responded to light touch but had not consumed any food for 5 days. After five days of not feeding, these larvae were removed from the experiment and freeze-killed.

Clothianidin had a significant effect on larval size (Figure 3, RM ANOVA, $F_{4, 126}$=3.632, p= 0.008) with a significant pairwise difference between the control and 15ppb (Dunn's with Bonferroni correction, p=0.024), indicating that 15ppb is the minimum growth inhibition concentration. The growth rate of larvae in the control, 5ppb, 15ppb and 50ppb all followed similar patterns of growth over time with the control group exhibiting the fastest initial growth rate; the growth of individuals in the 500ppb treatment group was delayed in the initial stages but appears to exhibit a faster growth rate from day 9.

There was no significant effect of treatment on development time (start of the experiment to the pupal stage) ($F_{4, 91}$= 0.326, p=0.860, GLM), pupal weight ($F_{4, 91}$ = 0.797, p= 0.530, GLM), adult weight ($F_{5,90}$=0.394, p=0.852) or the duration of the pupal stage ($F_{5,90}$ = 1.759, p=0.129, GLM). The highest levels of pupation were observed in the control group (26) with the lowest observed in 50ppb (17) (Table 2). Median time to pupation in the control and 5ppb groups was 3 days shorter (33 ± 4.58; 33 ± 10.1) than those in the 15ppb, 50ppb and 500ppb groups (36 ± 3.16; 36 ± 6.48; 36) (Table 2).

Discussion

This study gives a first estimate of the high levels of neonicotinoid contamination in margin vegetation (from plants grown specifically to be attractive to pollinators) after only one growing season of typical use. In the field, clothianidin was found in both *L. corniculatus* and *T. hybridum,* but contamination levels varied markedly between the two species, with much higher levels of clothianidin detected in *T. hybridum.* The maximum concentration of clothianidin we found in *T. hybridum* (48ppb) is higher than that which has been found in previous studies of the contamination of non-target vegetation by clothianidin, though different plant species were studied (Rundlöf *et al*. (2015): field border plants, ≤ 2 days after sowing of treated crops: 1.2 ± 0.8ppb, ≤ 2 weeks after sowing: 1.0 ± 0.8ppb; Pecenka and Lundgren, (2015): milkweed, 1.14 ± 0.10 ppb clothianidin, maximum of 4 ppb).

Our results suggest that the contamination of vegetation by clothianidin may be more likely when wildflowers are planted directly into soil that has previously supported a crop grown from neonicotinoid treated seed, compared to contamination levels of a flower margin grown alongside a treated crop, presumably from lateral movement of neonicotinoids in the soil over time. The differences in levels of contamination between *T. hybridum* compared to *L. corniculatus* are likely to be due to a difference in physiologies as levels in *T. hybridum* were consistently higher than *L. corniculatus* sampled from the same site. The range of concentrations amongst the samples is probably due to the heterogeneity in soil properties and environmental effects along the margin (e.g. degree of slope, humidity, sunlight exposure, microbial communities and organic matter content) causing a differential uptake of the active ingredients by the plants.[11,28] These results are comparable to Botías *et al*. (2016) who found that concentrations of neonicotinoids differed widely between wild plant species adjacent to a treated crop, even in the same margin.

Vegetation sampled from plants 10 months after sowing with Redigo Deter© treated wheat (Field A) seed frequently also contained thiamethoxam. With no history of thiamethoxam use on the farm, it is possible this compound has been introduced to the soil via dust drift or soil dust from a neighbouring farm[29], from contaminated machinery or seeds, **or from contaminated soil water from a neighbouring farm**. Similarly, Botías et al. (2015) found thiacloprid in wildflower samples from several farms with no history of its use. This highlights the likelihood of wide-scale contamination so that even strips planted for the benefit of pollinators do not provide safe refuge from chemicals[28]. Sampling occurred at the later stages of the margin's seasonal lifespan (a week before the margin was to be mown) so it is possible that clothianidin levels were higher earlier on in the season before the chemical had been metabolised in the plant. **However, due to the small sample size involved in the field trial, these results provide a proof-of-concept for the risk of non-target vegetation contamination under different growing regimes. Regardless,** The field study confirms the reality that beneficial herbivorous insects will be exposed to clothianidin in field conditions.

This evidence of long-term persistent contamination in the field indicates that neonicotinoid levels used in the experimental chronic exposure of the larvae in this laboratory based investigation are a true reflection of field conditions. **Survival was significantly lower when exposed to 500ppb thiamethoxam, but this concentration exceeds that likely to be found in the field.** ~~A lethal effect of clothianidin on *P. icarus* larvae was evident, there was no linear relationship between dose and mortality~~. Across a range of insects tested, LD50's for non-targets range from 3.7ppb to as much as 81ppb[19], for example the levels we found in the marginal vegetation samples overlap with the reported 36hr LC_{50} value of 15.63 for *Danaus plexippus*[11].

In this study, we found clothianidin negatively affected early stage development of the larvae, with larval size being reduced by the presence of field realistic levels. Despite being unable to pinpoint development times to exact instars, it seems possible that instars varied in their sensitivity to clothianidin over time, with larvae being affected by the toxin during a specific period, and those

making it past this early stage being more likely to survive. Larvae of *D. plexippus* (Nymphalidae) also take longer to develop, with first instars having reduced body weight and reduced length when exposed to clothianidin in their diet; this effect was not detected in second instars[19]. This variation in the sensitivity of larval stages has also been observed in *Cydia pomonella* (Tortricidae), with first instar caterpillars being more than 100 times more sensitive than fifth instar caterpillars.[18] It seems likely that this time dependant aspect of the early-stage development is due to the changing physiology of the developing insect at different instars.[30] Negative effects on early larval growth can make a population more likely to succumb to stressors like parasites, which could therefore amplify population declines.[19]

The contamination of larval food sources has a detrimental effect on early stage development and our data add to growing evidence that the pollution of non-target vegetation may be detrimental to early herbivorous larval stages. Overall, clothianidin had no effect on time to pupation due to apparent compensatory growth in the later stages. Costs for compensatory, accelerated growth periods include increased rates of mortality and decreased longevity.[31] Further work is required to investigate if this compensatory growth phase had a negative impact on the resulting adult as lower fecundity is associated with decreased pupal development time in some Dipteran species.[32]

As well as considerable variation in the sensitivities of species to neonicotinoids, and the different toxicity of the neonicotinoid compounds, there are also many documented studies showing neonicotinoid resistance in ecologically relevant species.[17] Encouragingly, the survival of some larvae in the 500ppb group to adulthood is a strong indication that there exists a physiological ability to cope with the toxin at the larval stage. Our study was not designed to calculate an LC_{50} but would suggest that for *P. icarus* the value is over 500ppb for clothianidin.

~~A recent correlational study modelled the population indices of 17 widespread butterfly species that commonly occurred at farmland sites against the number of hectares of farmland where neonicotinoid pesticides are used.[3] Fifteen of the seventeen species studied showed negative~~

~~correlations with neonicotinoid usage. *P. icarus* underwent a 30% decline from 2000 to 2009 but showed a weak negative relationship with hectares of neonicotinoid usage.[3] This result combined with those of our study demonstrates that *P. icarus* may be less sensitive than other butterflies, but further experimental studies to determine the toxicity and effect of neonicotinoids on *P. icarus* adults are required.~~

~~Much of the research on the impact of neonicotinoids on herbivorous larvae have short exposure durations, the maximum being 7 days, and the longest clothianidin exposure in the available literature is 36hr.[19] It has been argued that studies with short exposure durations may miss the critical period where a time cumulative effect of the toxin is detected.[30] This study followed the larvae from eggs through to metamorphosis and emergence, with chronic exposure occurring from when larvae were one week old. Due to the larvae's small size it is unlikely that they will disperse from the field margin,[33] and therefore we would expect chronic oral exposure.~~

~~Previous works utilised a leaf-dip assay or an agarose gel containing the contaminant,[19,34] but due to the nature of the exposure route, larvae can be exposed to the contaminant by contact residues as well as orally. Our novel contamination method ensured that the larvae was exposed to clothianidin only via the consumption of the foodstuff, so we can be sure that we are reporting the effects of oral toxicity only; it also mimics natural exposure methods much closer than a leaf bioassay or artificial lepidopteran food. The contamination technique resulted in vegetation that was very closely matched in neonicotinoid concentration to that of the watering solution, which confirms the levels the caterpillars were exposed to.~~

Overall, we show that growing a crop of neonicotinoid seed-dressed autumn-sown wheat results in the contamination of marginal vegetation growing alongside the crop and the soil so that subsequent, non-target plants grown in this soil become contaminated. We found high levels of contamination after 21 months in marginal strips grown for pollinators, with evidence of unintentional contamination from external sources. We detected ranges of 0.2ppb to 48ppb of

clothianidin in margin vegetation after only 10 months of typical use. Further work is required to establish the range of levels of contamination found in different field scenarios; contamination is likely to depend on many factors, including application rates, soil type, slope, crop type etc.

The presence of neonicotinoids in marginal vegetation is a cause for concern, as these levels can overlap with LC_{50} values for non-target, important insects.[12] There is a pressing need to mitigate against non-target contamination by neonicotinoids in the field, especially since these margins are grown to boost pollinator populations, to attract natural enemies of arthropod pests,[11] and to provide a food source for herbivorous species and larvae.

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